Rapid Diagnosis of Rotavirus by an Enzyme-Linked Immunosorbent Assay (ELISA) in an Outbreak of Neonatal Diarrhea in Dairy Calves

S. J. McGuire, *M.S.*
A. E. Castro, *D.V.M., Ph.D.*
Oklahoma Animal Disease Diagnostic Laboratory
College of Veterinary Medicine
Oklahoma State University
Stillwater, Oklahoma 74078

SUMMARY

An outbreak of neonatal calf diarrhea (NCD) occurred in a group of 20 newborn calves in a dairy herd of 260 Holsteins. Feces from 10 of 13 calves were positive or suspicious for the group-specific (gs) antigen of rotavirus by an enzyme-linked immunosorbent assay (ELISA). Feces from 4 cows including the dam of 1 clinically ill calf were negative for rotavirus by the ELISA. In 1 of 3 calves, rotavirus was identified by ELISA in fluids obtained from cell cultures inoculated with feces. A serologic profile of sera from 7 calves and 5 cows revealed no other significant data. The rapid detection of rotavirus in the herd by ELISA within 24 hours after collection of fecal samples in the outbreak of NCD brought about a treatment regimen of colostral feeding and a vaccination program for rotavirus for newborn calves.

INTRODUCTION

Neonatal calf diarrhea (NCD) or "calf scours" causes extensive calf and economic losses in dairy and beef herds (1, 2, 3, 4). The etiology of NCD is complex and *Escherichia coli,* coronavirus, rotavirus and cryptosporidia have been isolated from clinically ill animals (1, 4, 5). Rotaviruses have also been implicated as major pathogens in diarrheas and gastroenteritis of children (6, 7, 8). The diarrheas and associated gastroenteritides caused by rotaviruses appear to be primarily disease problems of newborn or young individuals (7, 8, 9).

The name "rotavirus" was suggested by Flewett and coworkers (10) because of the spoke and "wheel-like" appearance of viral particles seen by electron microscopy (EM). Rotaviruses are double-stranded RNA viruses, 70 nm in diameter, contain two protein coats (capsids), and belong to the family Reoviridae (8, 11). A group-specific (gs) antigen located in the inner capsid of the virion is shared by all rotaviruses and this antigen is the basis of several diagnostic tests. A type-specific neutralizing antigen is located on the outer capsid of the virion (11).

The rotaviruses are shed in high concentrations in feces of affected animals, thus detection of virus in fecal material can be done by electron microscopy, immunofluorescence, counter immunoelectro-osomophoresis, viral isolation in cell cultures and radioimmunoassay (RIA) (4, 7). Certain of these diagnostic techniques have also been used to detect antibodies to rotavirus in various species (12, 13). Recently, an enzyme-linked immunosorbent assay (ELISA) has been developed which detects the gs antigen of rotavirus in feces from calves (12) and humans (14, 15, 16).

In this report, we present data concerning an outbreak of NCD in a Holstein dairy herd and the rapid diagnosis of rotavirus using a commercial immunoassay.

MATERIALS AND METHODS

History of Dairy Herd: A dairy herd of the Holstein breed consisted of 120 cows, 110 heifers and 20 calves. The milk production in the herd averaged 46.2 pounds of milk per day. The herd was fed a ration containing ground corn, yeast, minerals, salt and sodium bicarbonate; also, each cow was fed an average of 20 pounds of hay (alfalfa and prairie) per day. Calves born into the herd were from natural breeding and were fed colostrum after birth. The herd had been vaccinated for Leptospira and calves with scours had been fed pooled colostrum supplemented with 1% tamed iodine and gentamicin. Newborn calves were housed in separate stalls in an enclosed calf barn.

Clinical specimens: Blood samples were collected from 5 cows and 7 calves. Sera were tested for the presence of antibodies to Leptospira, Brucella, infectious bovine rhinotracheitis (IBR) virus, bovine viral diarrhea (BVD) virus, bluetongue virus (BTV), bovine leukemia virus (BLV), bovine coronavirus and bovine rotavirus (Table 1).

TABLE 1: A Selected Serologic Profile of Cattle Tested From Herd

Animal ID Number	Age***	TYPE OF SEROLOGIC TEST							
		Leptospira[a]	IBR[b]	BVD[c]	Rota[c]	Corona[c]	Brucella[d]	BTV[e]	BLV[e]
		Serum-dilution Titers							
1	A	<200	16	<4	320	≥1280	0	+	0
2	A	<200	<4	≥4	80	≥1280	0	+	0
3*	A	<100	8	<4	320	≥1280	0	+	+
4**	A	<100	<4	<4	80	≥1280	0	+	0
5	A	<100	8	≥4	320	≥1280	0	0	0
6	C	<100	<4	<4	≥20	<20	0	0	0
7	C	<100	<4	≥4	≥20	≥20	0	+	0
8	C	<100	ND	≥4	≥20	≥20	0	+	+
9	C	<200	ND	≥4	≥20	≥20	ND	ND	ND
10	C	<400	<4	≥4	≥20	≥20	0	+	+
11	C	<100	<4	≥4	≥20	≥20	0	ND	ND
12	C	<100	<4	≥4	≥20	≥20	0	0	0

*Dam of calf no. 13; **Dam of calf no. 9; ***Adult (A) = >2 years of age, Calf (C) = less than 3 months of age

0 = negative antibody, + = positive antibody ND = not done

[a]microagglutination-lysis test for serotypes: L. canicola, L. grippotyphosa, L. hardjo, L. icterohemorrhagiae, L. pomona;

[b]serum-neutralization test; [c]indirect fluorescent antibody test; [d]agglutination test; [e]agar-gel immunodiffusion test.

Fresh fecal samples collected from 13 calves and 4 cows were tested for the presence of rotavirus group-specific (gs) antigen by an enzyme-linked immunosorbent assay. Fecal samples from 3 calves positive for rotavirus by ELISA were passaged onto cell cultures using a trypsin treatment procedure (17). A direct immunofluorescence test was used on the inoculated cell cultures for the detection of rotavirus.

Enzyme-linked immunosorbent assay (ELISA): The ELISA procedure used is diagrammatically outlined in Figure I and 2. The commercial immunoassay[a] was done in test tubes using plastic (polystrene) beads coated with guinea pig antibody to rotavirus. The fecal specimens to be tested were diluted 1:5 (w/v) in phosphate buffered saline, pH 7.4, and incubated with beads for 3 hours at 45° C. Following incubation, the inocula were removed and the beads were washed 4 times with distilled water. A conjugate consisting of anti-rotavirus antibody and horseradish peroxidase enzyme was added to each of the beads and allowed to react for 60 minutes at 45° C, then each bead was washed 6 times with distilled water. The reacted beads were transferred to new test tubes and hydrogen peroxide and a color indicator o-phenylenediamine-2 HCl (OPD) were added. Following an incubation of 15 minutes in the dark at ambient temperature, the colorimetric reaction was read qualitatively (by a color chart) and quantified spectrophotometrically[b].

Cell cultures for viral isolation: Bovine cell cultures were prepared by trypsinization of kidneys (BEK) from bovine fetuses (13 to 15 cm long).[c] The cells were grown in 75 cm^2 flasks supplemented with Dulbecco's medium containing 200 mg of gentamicin/ml and 10% bovine fetal serum (BFS).

Embryonic rhesus monkey kidney cells (MA-104)[d] were grown in F_{15} medium supplemented with 10% BFS and 200

[a] "Rotazyme ®", Abbott Laboratories, Diagnostic Division, Chicago, Illinois 60064.

[b] Spectrophotometer Model 700, Bausch and Lomb Company, Rochester, New York 14625

[c] Courtesy of Mikkelson Beef, Inc., Oklahoma City, Oklahoma

[d] Cell cultures of MA-104 were kindly provided by Dr. Robert W. Fulton, College of Veterinary Medicine, Oklahoma State University, Stillwater, Oklahoma 74078

TABLE 2: Detection of Rotavirus by ELISA in Feces From Cattle in Herd

Animal ID No.	Age**	Results of ELISA	Clinical Data on Animal
1	A	0	Normal pregnant cow
14	A	0	Normal cow
15	A	0	Normal cow
22	A	0	Normal cow
6	C	±	Calf received rotavirus vaccine; anorectic, slightly dehydrated; feces were semisolid.
8	C	0	Recovered from NCD
* 9a	C	+	Anorectic, slightly dehydrated; acute case.
b	C	0	Recovered from NCD
* 10a	C	+	Anorectic, slightly dehydrated; acute case.
b	C	±	Recovered from NCD
* 11a	C	±	Anorectic, slightly dehydrated, acute case.
11b	C	0	Recovered from NCD
12	C	+	Diarrheic feces, slight dehydration; acute case.
13	C	+	Diarrheic, anorectic, slightly dehydrated, acute case.
16	C	0	Recovered from NCD
17	C	±	Prior history of diarrhea, recovered from NCD
18	C	+	Diarrheic feces; acute case.
19	C	0	Diarrheic feces, recovered from NCD
20	C	+	Diarrheic feces, acute case.
21	C	±	Diarrhecic feces, recovered from NCD

*2 samples collected 1 month apart **A = adult 2 years of age, C = calves < 2 months of age

0 = negative + = positive ± = suspicious

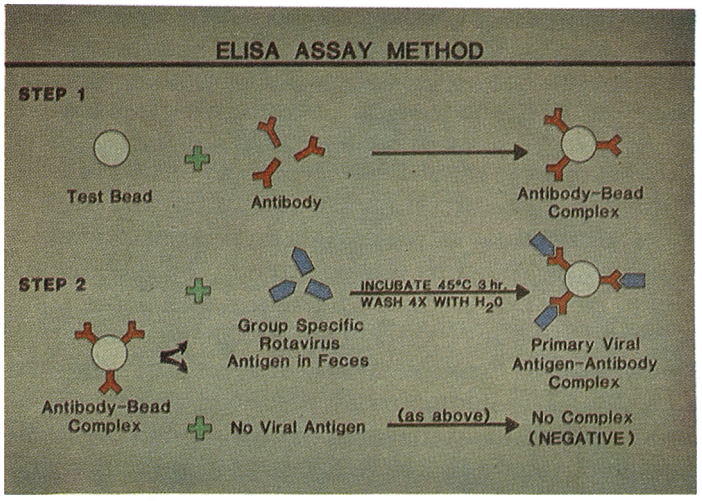

Figure No. 1

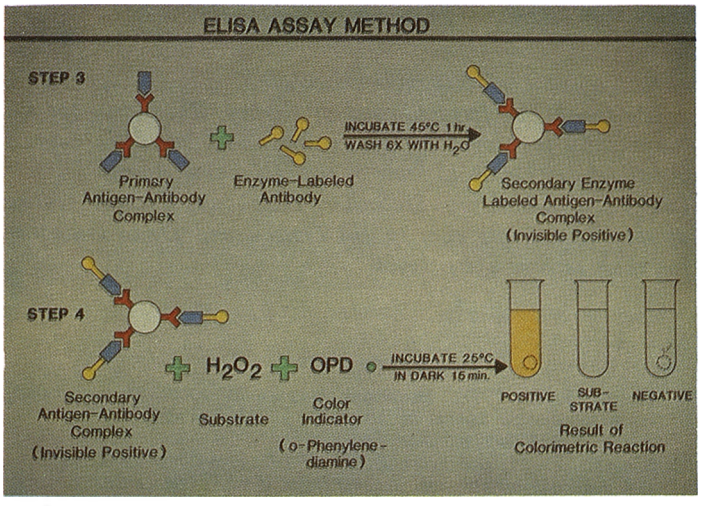

Figure No. 2

mg of gentamicin/ml. For propagation of virus, cell cultures were maintained in F_{15} medium containing 2% BFS.

Direct fluorescent antibody conjugates for rotavirus were obtained from the National Veterinary Services Laboratories at Ames, Iowa.

RESULTS

In the dairy herd of 150 cows and 110 heifers, none of the cattle were ill or scouring during the outbreak of NCD. Sera from five adult cows tested had antibodies to rotavirus with immunofluorescent titers between 80 to 320 (Table 1). A serologic survey (Table 1) for antibodies to other bacterial and viral pathogens was unremarkable. Freshly collected feces from 4 cows were negative for rotavirus by the ELISA test (Table 2).

The clinical signs observed in the calves with "scours" included a profuse, yellow-brown watery diarrhea. These affected calves were anorexic and slightly-to-moderately dehydrated. Fecal material was pasted onto the hair of the perineum. Some deaths had occurred in calves with similar signs during prior parturitions. All of the 13 diarrheic calves recovered uneventfully following antibiotic therapy and colostral feedings. One calf was vaccinated at birth with a rotavirus vaccine and the calf developed slight scours but also recovered.

Serum antibody to rotavirus was detected in 7 of 7 calves tested (Table 1). The serologic profile for other pathogens in these calves was unremarkable. However, rotavirus was detected by ELISA in the feces of 10 of 13 diarrheic calves (Table 2). Cow no. 3 (Table 1) the dam of calf no. 13 (Table 2) had antibodies to rotavirus and coronavirus; however, the calf had not nursed its mother. Cow no. 4 (Table 1) the dam of calf no. 9 (Table 2) also had antibodies to rotavirus and coronavirus, but the nursing history was unknown. Two fecal samples from calf no. 9 taken at 2 and 3 months of age revealed that the antigens of rotavirus were detectable when the ELISA was applied at 60 days but the virus was not detected in the sample taken at 90 days.

Viral isolation attempts were made in BEK and MA-104 cell cultures from 3 of 10 calves positive for rotavirus by ELISA. The sample from calf no. 13 was placed on both BEK and MA-104 and 6 passages were made in BEK and 2 passages in MA-104. Cells inoculated from these passages were tested by immunofluorescence and were found negative for rotavirus. However, clarified supernatant from passage 6 in BEK and 2 MA-104 cells tested positive for gs antigen of rotavirus. The other 2 fecal specimens were negative after passage in BEK cells.

A pure culture of *Escherichia coli* was isolated and identified from the feces of calf no. 13. Further bacteriology on the other calves was not attempted because the other calves were placed on antibiotic therapy.

DISCUSSION

The isolation of a reovirus-like agent from calves with NCD (18, 19) led to further studies (19, 20, 21) which identified the virus isolated as rotavirus. Rotaviruses are ubiquitous since isolations from lambs, foals, rabbits, deer, antelope, neonatal mice (7, 8) and human infants (6, 10, 17) have been documented.

The mechanism of NCD is complex; however, from investigations in animals and humans, a common description of the pathogenesis of rotavirus has emerged. Rotaviruses affect primarily epithelial cells (enterocytes) which cover the villi of the small intestine (7, 19). Studies with humans (7) indicate that in infected children, desquamated columnar epithelial cells are replaced by cuboidal epithelium and the microvilli of the absorptive epithelial cells are distorted. The disaccharidase enzymes are decreased in infected epithelial cells leading to a decrease in the absorptive surface of the small intestine (7, 22). In part, this decreased absorptive phenomenon accounts for the diarrhea seen in infections with rotavirus (7, 22). Other contributory conditions seen appear to be due to bacterial infections (*Escherichia coli*) through the denuded villi (2). The incubation time for rotavirus in most species varies from several hours to several days (4, 7, 19).

In calves, NCD usually occurs during the first days of life (19), however, the virus can also infect adult cattle but the disease is not as severe (8). In children, the disease is pri-

marily seen between 6 months to 1 year of age with a greater incidence in winter months (8). Protection in calves appears to be derived from the presence of protective antibodies present in colostrum (20) since calves with elevated serum titers (40 to 160) to rotavirus developed diarrhea; but if calves were fed colostrum that contained specific immunoglobulins at levels of 30 mg/ml or greater, diarrhea did not develop (21). Protection to rotavirus infection in calves diminished when they were fed colostrum in which immunoglobulin levels had declined (9). Vaccination studies in calves (23) indicated that serum circulating antibody was not indicative of protection. In the preceding study (23), results suggested that exposure of the intestinal epithelium to a modified live rotavirus vaccine reduced the incidence of diarrhea in calves and furthermore protection did not appear to be immunologically mediated.

In our study, the presence of circulating serum antibody in diarrheic calf no. 13 also suggested the lack of protection by serum antibodies. Other investigators have also reported diarrhea in calves (20) and children (6) where elevated levels of serum immunoglobulins to rotavirus were present. The finding that cow no. 3, mother of calf no. 13, had antibodies to rotavirus indicates exposure of dam to virus, but its newborn calf had "scours" and not nursed which suggests the possible infection of the fetus *in utero* and the ability of rotaviruses to cross the placenta. Along similar lines (8), antibodies to rotavirus were detected in 46% of the sera from 69 bovine fetuses. Colostral ingestion by the calves in this herd appeared to decrease the severity of diarrhea and enhanced recovery of the diarrheic calves. This observation indicated protective levels of immunoglobulins in the colostrum that was ingested. Therefore, as in previous studies (20), our results suggested that circulating antibodies to rotavirus in cows are not indicative of protection. Protection against infection by rotavirus is reflected more accurately by the level of antibody in the colostrum or milk being fed. Since the protective effect of immunoglobulins in colostrum has been reported to drop within 5 days (9), a calf should receive colostrum or rotavirus vaccine as soon as born (9, 23).

The source of the current outbreak of NCD was not known but we did not test sufficient adult cattle in the herd to eliminate the possibility of an adult "carrier". In certain calf diarrheas, the source of rotavirus appeared to be the dam since rotavirus has been detected in both dam and offspring (20, 21). This route of transmission did not appear to be the source of this outbreak of NCD. Because of our findings, a vaccination program for rotavirus was recommended for adults and newborns in the herd and the feeding of colostrum to newborn calves be continued. There have been no additional outbreaks of NCD in the herd.

Diagnosis of rotavirus has been primarily by detection of virus by electron microscopy (EM) (3, 6, 22, 24, 25). Virus isolation and identification has also been accomplished by the treatment of feces with trypsin (17) or chymotrypsin (25). The ELISA developed for the detection of virus in feces of calves and antibody to rotavirus in microplates had a sensitivity of 20 to 30 ng/ml of viral protein (12). In diarrheic feces from calves rotavirus was detectable at a 1:100,000 dilution (12). Furthermore, it has been estimated that approximately 10^{10} viral particles per gram of feces may be present in diarrheic individuals (8). The sensitivity of the ELISA for the detection of rotavirus in feces from calves was determined to be 100 fold higher than by EM (14). Therefore, the ability to detect viral antigens in feces from various species by the ELISA is based on the presence of a gs antigen within the virus core (8, 11) and the concentration of viral particles.

In this study of NCD, we identified rotavirus in feces of diarrheic calves using a commercial immunoassay. Also, rotavirus was detected in the supernatant fluids from cell cultures inoculated with feces from a rotavirus-positive calf. This ELISA has been routinely used to detect rotavirus in feces from children (15, 26). The immunoassay described avoids specialized equipment as required by EM, poses no radiation biohazard as in the RIA test, requires only visual reading and the reagents are stable during storage (15). The ease of the ELISA will enhance the rapid diagnosis of diarrheas in cattle caused by rotaviruses.

Acknowledgements

We thank Mr. Jerry Elwood of Carney, Oklahoma, for his cooperation in providing the clinical specimens. We express our thanks to Dr. E. L. Stair of the Oklahoma Animal Disease Diagnostic Laboratory, Stillwater, Oklahoma, for the review of the manuscript; also, to Jill Dotson and Linda Guenther for their technical assistance.

REFERENCES

1. House, J. A. 1978. Economic impact of rotavirus and other neonatal disease agents of animals. JAVMA 173:573-576. Assoc. 173:573-576.
2. Schlafer, D. H. and Scott, F. W. 1979. Prevalence of Neutralizing antibody to the calf rotavirus in New York cattle. Cornell Vet. 69:262-271.
3. England, J. J., Frye, C. S. and Enright, E. A. 1976. Negative contrast electron microscopic diagnosis of viruses of neonatal calf diarrhea. Cornell Veterinarian 66:172-182.
4. Kahrs, R. F. Rotavirus associated with neonatal diarrhea in: Viral diseases of cattle 1981. Chapter 25, p. 225-233, The Iowa State University press, Ames, Iowa.
5. Stair, E. L., Rhodes, M. D., White, R. G., and Mebus, C. A. 1972. Neonatal calf diarrhea: Purification and electron microscopy of a coronavirus-like agent. Amer. J. Vet. Res. 33:1147-1156.
6. Kapikian, A. Z., Kim, H. W., Wyatt, R. G., Cline, W. L., Arrobio, J. O., Brandt, C. D., Rodriquez, W. J., Sack, D. A., Chanock, R. M. and Parrott, R. H. 1976. Human reovirus-like agent as the major pathogen associated with "winter" gastroenteritis in hospitalized infants and young children. The New England J. of Med. 294:965-972.
7. Steinhoff, M. C. 1980. The Journal of Pediatrics 96:611-622, Medical Progress Rotavirus: The first five years.
8. McNulty, M. S. Review Article Rotaviruses. 1978. J. Gen. Virol. 40:1-18.
9. Acres, S. D. and Babiuk, L. A. 1978. Studies on rotaviral antibody in Bovine serum and lacteal secretions, using radioimmunoassay. J. Amer. Vet. Med. Assoc. 173:555-559.
10. Flewett, T. H., Bryden, A. S. Davies, H., Woode, G. N. Bridger, J. C. and Derrick, J. H. 1974. Relation between viruses from acute gastroenteritis of children and newborn calves. The Lancet ii :61-63.
11. Bridger, J. C. 1978. Location of type-specific antigens in calf rotaviruses. Journal of Clinical Microbiology 8:625-628.
12. Scherrer, R. and Bernard, S. 1977. Application d'une technique immunoenzymologique (ELISA) a la detection du rotavirus

bovin et des anticorps diriges contre lui. Ann. Microbiol. (Insti. Pasteur 128A:499-510.
13. Babiuk, L. A., Acres, S. D., and Rouse, B. T. 1977. Solid-phase radioimmunoassay for detecting bovine (neonatal calf diarrhea) rotavirus antibody. Journal of Clinical Microbiology 6:10-15.
14. Ellens, D. J. and deLeeuw, P. W. 1977. Detection of infantile gastroenteritis virus (rotavirus) by ELISA. Lancet i :1363-1364.
15. Yolken, R. H., Kim, H. W., Clem, T., Wyatt, R. G., Chanock, R. M., Kalica, A. R. and Kapikian, A. Z. 1977. Enzyme-linked immunosorbent assay (ELISA) for detection of human reovirus-like agent of infantile gastroenteritis; The Lancet ii :263-266.
16. Yolken, R. H., Barbour, B. A., Wyatt, R. G. and Kapikian, A. Z., 1978. Immune response to rotavirual infection - Measurement by enzyme immunoassay JAVMA 173:552-554
17. Wyatt, R. G., James, W. D., Bohl, E. H., Theil, K. W., Saif, L. T,, Kalica, A R., Greenberg, H. B., Kapikian, A. Z., and Chanock, R. M. 1980. Human rotavirus type 2: Cultivation in vitro. Science 207:189-191.
18. Mebus, C. A., Underdahl, N. R., Rhodes, M. B., and Twiehaus, M. J. 1969. Calf diarrhea (scours): Reproduced with a virus from a field outbreak. University of Nebraska Res. Bull 233:-1-16.
19. Mebus, C. A., Stair, E. L., Underdahl, N. R., and Twiehaus, M. J. 1971. Pathology of neonatal calf diarrhea induced by a reo-like virus. Veterinary Pathology 8:490-505.
20. Woode, G. N. and Bridger, J. C. 1976. Rotavirus in calves. Veterinary Record 99:322-333.
21. McNulty, M. S., McFerran, J. B., Bryson, D. G., Logan, E. F. and Curran, W. L. 1976. Studies on rotavirus infection and diarrhea in young calves. Veterinary Record 99-229-330.
22. Flewett, T. H. and Woode, G. N. 1978. The rotaviruses Brief view. Archives of Virology 57:1-23.
23. Mebus, C. A., White, R. G., Bass, E. P. and Twiehaus, M. J. 1973. Immunity to neonatal calf diarrhea virus JAVMA 163:-880-883.
24. Bishop, R. F., Davidson, G. P., Holmes, I. H. and Ruck, B. J. 1974. Detection of a new virus by electron microscopy of faecal extracts from children with acute gastroenteritis. The Lancet i :149-151.
25. Rhodes, M. B., Stair, E. L., McCullough, R. A., McGill, L. D. and Mebus, C. A. 1979. Comparison of results using electron microscope, immunodiffusion and fluorescent antibody analyses to detect rotavirus in diarrheic fecal samples of calves. Can. J. Comp. Med. 43:84-89.
26. Yolken, R. H. and Leister, F. J. 1981. Evaluation of enzyme immunoassay for the detection of human rotavirus. Journal of Infectious Diseases, 144:379.